Cell PRESS
Cancer Cell
Article

PML/RARA Oxidation and Arsenic Binding Initiate the Antileukemia Response of As_2O_3

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DOI 10.1016/j.ccr.2010.06.003

SUMMARY

As_2O_3 cures acute promyelocytic leukemia (APL) by initiating PML/RARA oncoprotein degradation, through sumoylation of its PML moiety. However, how As_2O_3 initiates PML sumoylation has remained largely unexplained. As_2O_3 binds vicinal cysteines and increases reactive oxygen species (ROS) production. We demonstrate that upon As_2O_3 exposure, PML undergoes ROS-initiated intermolecular disulfide formation and binds arsenic directly. Disulfide-linked PML or PML/RARA multimers form nuclear matrix-associated nuclear bodies (NBs), become sumoylated and are degraded. Hematopoietic progenitors transformed by an As_2O_3-binding PML/RARA mutant exhibit defective As_2O_3 response. Conversely, nonarsenical oxidants elicit PML/RARA multimerization, NB-association, degradation, and leukemia response in vivo, but do not affect PLZF/RARA-driven APLs. Thus, PML oxidation regulates NB-biogenesis, while oxidation-enforced PML/RARA multimerization and direct arsenic-binding cooperate to enforce APL's exquisite As_2O_3 sensitivity.

INTRODUCTION

The PML/RARA oncoprotein induces acute promyelocytic leukemia (APL) through homo- and heterodimerization with RXRA, leading to deregulation of transcription, differentiation arrest, and enhanced self-renewal of leukemia-initiating blast cells (LIC)(Kwok et al., 2006; Martens et al., 2010; Mikesch et al., 2010; Zeisig et al., 2007; Zhu et al., 2007). As_2O_3 cures APL through degradation of PML/RARA and the eradication of LIC (Ghavamzadeh et al., 2006; Kogan, 2009; Mathews et al., 2006; Nasr et al., 2008; Zhu et al., 2002). Arsenic atoms bind vicinal cysteines of many target proteins, which results, among others, in the poisoning of phosphatases or mitochondrial enzymes from the respiratory chain (Miller et al., 2002). Thus, like other metalloids, As_2O_3 elicits the formation of reactive oxygen species (ROS) (Kawata et al., 2007).

As_2O_3 elicits PML/RARA degradation by targeting its PML moiety. PML is an RBCC/TRIM protein that partitions between a large nucleoplasmic fraction and nuclear matrix-associated nuclear bodies (NBs) (Lallemand-Breitenbach and de Thé, 2010). The nuclear matrix is biochemically-defined by resistance to high salt/nuclease extractions. It was proposed to be associated with many processes (including DNA replication, transcription and epigenetic silencing). Yet, its exact nature and the existence of an actual morphologically definable structure has been a matter of dispute (Stuurman et al., 1992a; Zaidi et al., 2007). As_2O_3 elicits the transfer of the diffuse nucleoplasmic PML fraction toward the nuclear matrix and NBs, independently

Significance

Arsenic trioxide cures many patients with PML/RARA-driven leukemia by targeting its PML moiety. Arsenic induces PML/RARA degradation within distinct cellular domains, the nuclear bodies (NBs). PML or PML/RARA are sequentially modified by two peptides, SUMO and ubiquitin, which lead to their degradation by the proteasome. How arsenic initiates PML sumoylation was not understood. We demonstrate that arsenic induces PML/RARA crosslinking through both production of reactive oxygen species and direct binding. Disulfide-bound PML/RARA multimers then aggregate onto NBs and are degraded. Other oxidants trigger regression of PML/RARA-driven murine leukemias. Thus, PML/RARA oxidation contributes to its therapeutical effect.

of PML sumoylation, as demonstrated using a sumoylation-defective PML mutant, PML3KR (Lallemand-Breitenbach et al., 2001; Zhu et al., 1997). As_2O_3 also promotes sumoylation of the critical PML K160 residue (Lallemand-Breitenbach et al., 2001), which fosters recruitment of NB partners, including Daxx transcriptional repressor or the SUMO-dependent ubiquitin-ligase RNF4. In As_2O_3-exposed cells, RNF4 polyubiquitinates PML, ultimately triggering its degradation on NBs (Lallemand-Breitenbach et al., 2008; Tatham et al., 2008). Although key to its antileukemia activity, how As_2O_3 actually fosters PML matrix-association and sumoylation remains unknown.

RESULTS

Matrix-Associated PML Is Oxidized

The matrix-associated PML fraction is hypersumoylated (Lallemand-Breitenbach et al., 2001). Conversely, deletion of the coiled-coil of PML abrogates its association to the nuclear matrix, NB formation, and sumoylation (see Figure S1A available online). Thus, whereas As_2O_3 induces PML targeting to the matrix and NB formation independently of sumoylation, matrix association contributes to basal or As_2O_3-enhanced PML sumoylation. We therefore questioned how As_2O_3 could promote transfer from soluble diffuse nuclear PML to insoluble, matrix-associated NBs. As_2O_3 controls protein phosphorylation and ROS production. We could not confirm in our experimental system (not shown) that As_2O_3-induced PML phosphorylation by ERK1/2 controls PML sumoylation (Hayakawa and Privalsky, 2004). We then examined whether As_2O_3-induced ROS could promote NB formation through PML disulfide formation, as intermolecular disulfide bonds were implicated in stabilizing the nuclear matrix (Kaufmann et al., 1991; Stuurman et al., 1992b). We observed that treatment with DTT (a dithiol reductant) of nuclear matrix prepared in situ disrupted endogenous PML NBs, without affecting lamin-B staining (Figure 1A). Similarly, a 1 hr treatment of CHO cells stably expressing the PML-III isoform (CHO-PML) with the thiol alkylator N-ethylmaleimide (NEM) disrupted basal PML NBs (Figure 1B). Finally, most CHO-PML cells cultured for 10 days in the presence of the ROS scavenger N-acetyl cysteine (NAC) lost basal NB formation (Figure 1C). NBs were restored by a 3 day wash-out (not shown). Thus, cysteine residues and ROS regulate NB biogenesis.

NBs are matrix-associated domains. When analyzed in the absence of reducing agents (DTT or βME), the nuclear matrix fraction of transiently PML-transfected CHO cells entirely consisted of high molecular weight PML species (Figure 1D). DTT disrupts disulfide bridges and may also reduce SOH or SOS linkages. The observation that these high molecular weight PML complexes reversed to monomeric species upon reduction, strongly suggested that matrix-associated PML consists of intermolecularly disulfide-bound PML multimers. Accordingly, pretreatment of CHO cells with NAC prior to PML transfection led to a dramatic decrease in the abundance of the high molecular weight PML forms (Figure 1E). To formally demonstrate the existence of covalent PML multimers, we transiently expressed (His)x6-PML-V with or without CFP-PML-III (PML isoforms of different sizes; Figure 1F) in the presence of As_2O_3. Denatured whole-cell lysates were analyzed under nonreducing conditions with isoform-specific antibodies (Condemine et al., 2006). In cotransfected cells only, several, rather than one, high molecular weight complexes were detected with the PML-V-specific antibody (Figure 1F). Only the highest species reacted with anti-PML-III, implying that these were PML-III/PML-V multimers. PML-multimerization was also demonstrated by immunoprecipitating SDS-denatured lysates with anti-PML-III-specific sera, followed by guanidinium denaturation and His-purification of the (His)x6-PML-V-containing complexes. Under nonreducing conditions, only cells expressing both PML isoforms yielded high molecular weight PML-III- and PML-V-reactive conjugates, which shifted to the monomeric state after reduction (Figure S1B). Finally, mass spectrometric analysis of high molecular weight PML complexes purified under denaturing conditions from As_2O_3-treated cells consisted primarily of PML peptides (54%). Other detected proteins are most likely contaminants, given that the most abundant one represented <6% of the total peptides (Table S1). Collectively, these analyses strongly argue against heterodimer formation with a distinct protein. Thus, although we cannot rule out the existence of intramolecular disulfide linkages, these data indicate that intermolecular PML crosslinking by ROS-induced disulfides is associated with its presence in the nuclear matrix and with NB-formation.

We next investigated whether oxidants enhance disulfide-bound PML multimerization. A short exposure to therapeutic levels of As_2O_3 (Figure 1G) or to other oxidants, such as H_2O_2 or $CdCl_2$ (not shown), massively increased the amounts of covalent PML multimers in CHO cells transiently overexpressing PML (Figure 1G, not shown). In stable CHO transfectants, oxidants similarly induced PML multimerization, whereas arsenical also promoted PML sumoylation (Figure 1H). Formation of covalent multimers in transiently PML-transfected CHO cells was abrogated by pretreatment with NEM or NAC (Figures 1I and 1J). If PML covalent multimerization by ROS is indeed the primary event initiating As_2O_3 effects, other oxidants should mimic As_2O_3-enhanced NB biogenesis. Yet, strong oxidants ($CdCl_2$, H_2O_2…) disrupt NBs in cultured cells (reviewed in Lallemand-Breitenbach and de Thé, 2010), possibly because these agents fully oxidize some critical cysteines into cysteic acid, precluding disulfide formation and thus dissociating NBs (data not shown). We thus examined whether paraquat, an acute ROS inducer (Black et al., 2008), would regulate NB formation in vivo, a setting where ROS levels are likely more controlled. Although CHO-PML cells grown ex vivo displayed prominent NBs, those were barely detectable when these cells were grown as xenografts in nude mice (Figure 1K). This could suggest that the microenvironment (notably oxygen tension) in vivo does not favor NB formation, whereas ex vivo culture under hyperoxic conditions evokes NB aggregation. Critically, paraquat or As_2O_3 treatment of mice bearing CHO-PML xenografts elicited a dramatic increase in NB formation (containing both PML and its partner daxx) (Figure 1K). Western blot analysis under nonreducing conditions showed the occurrence of PML multimers in CHO-PML xenografts derived from oxidant-treated mice (Figure 1L). Enhanced NB formation by endogenous murine PML was also observed in bone marrow cells on treatment with paraquat (Figure 1K) or As_2O_3 treatment (not shown). Altogether, these data demonstrate that, in vivo, ROS inducers mimic As_2O_3 as to the regulation of PML oxidation, NB formation, and partner recruitment.

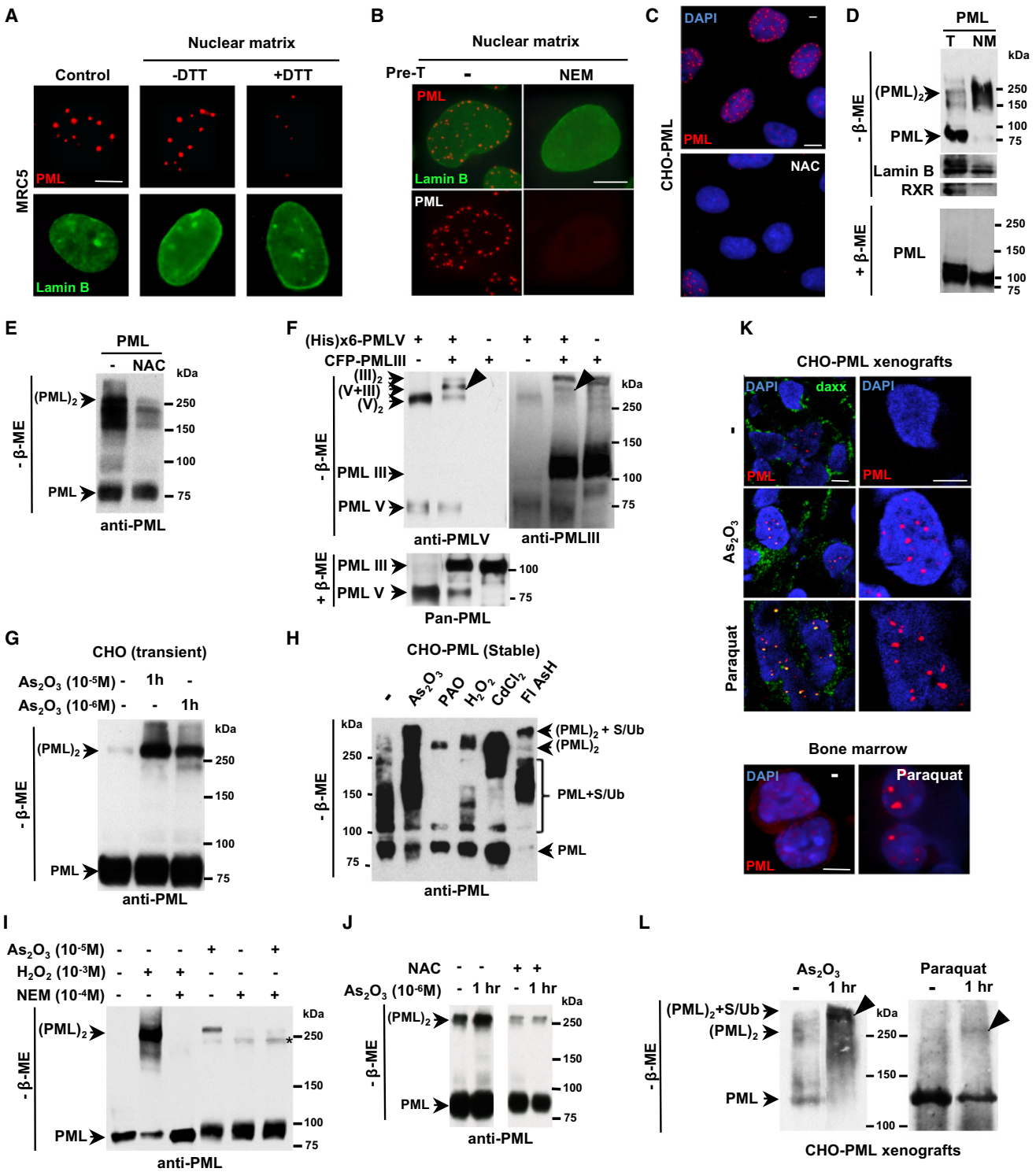


Figure 1. As_2O_3-Induced Disulfide Formation Targets PML to the Nuclear Matrix

(A) PML (red) and LaminB (green) staining of MRC5 cells. In situ nuclear matrix preparation were treated or not treated with DTT (10 mM, 10 min) prior to antibody labeling. The scale bar represents 5 μm.

(B) Prefixation treatment (pre-T) with NEM of CHO-PML cells prior to nuclear matrix preparation disrupts NBs. The scale bar represents 5 μm.

(C) Ten days of N-acetyl-cysteine (NAC) treatment decreases the number of PML bodies in CHO-PML cells. The scale bar represents 5 μm.

(D) Total cell lysates (T) or nuclear matrix (NM) fractions of CHO cells transiently transfected with PML were analyzed in nonreducing (−β-ME, top) or reducing (+β-ME, bottom) conditions.

(E) Pretreatment with NAC dramatically reduces formation of PML multimers in transiently transfected CHO cells.

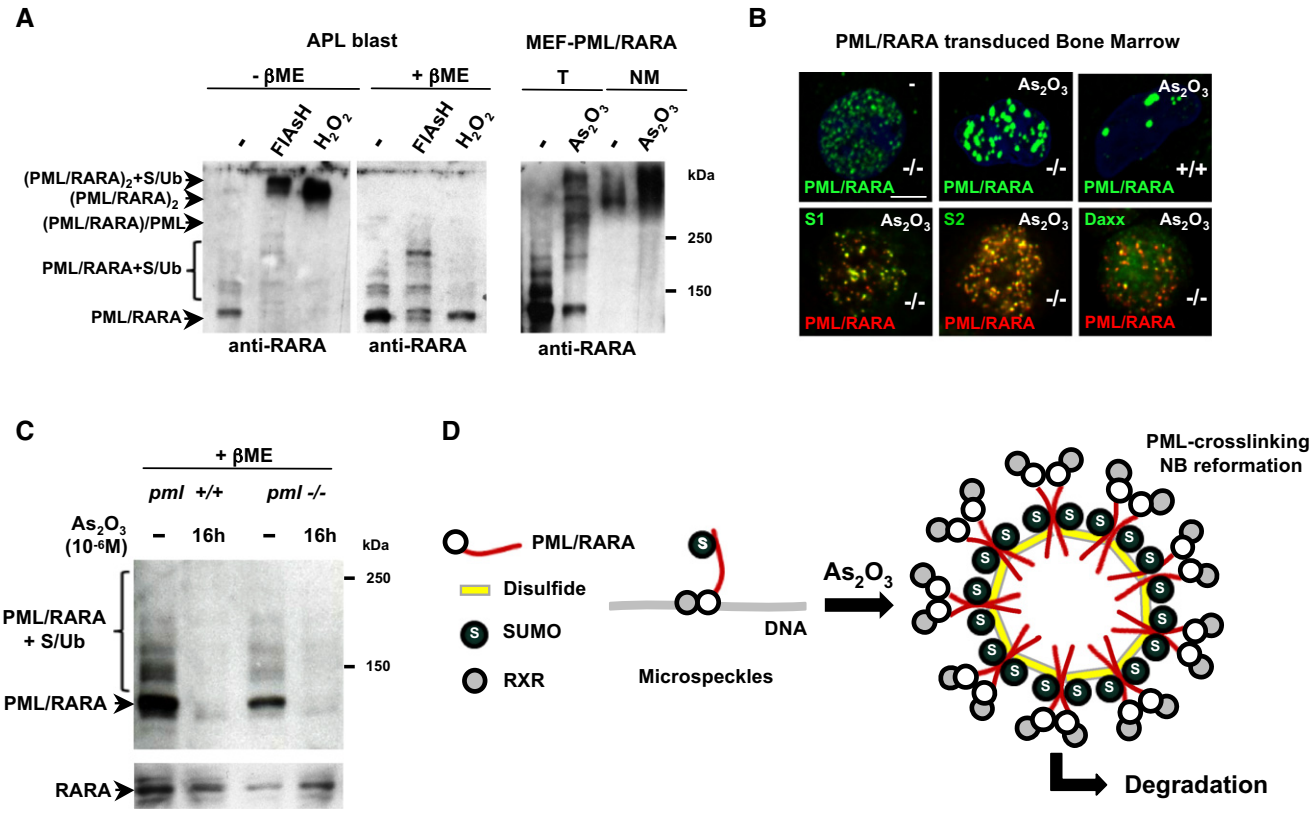


Figure 2. PML/RARA Oxidation Promotes NB Formation

(A) Shown on the left are oxidant-treated murine APL cells (from hPML/RARA transgenic mice), under reducing or nonreducing conditions. As shown on the right, As_2O_3 (10 μM, 1 hr) induces PML/RARA multimerization and attachment to the matrix. Cells were fractionated into total (T) and nuclear matrix (NM) extracts.

(B) The top row shows anti-hPML antibody staining of hPML/RARA-transformed mouse bone marrow progenitors treated or not treated with As_2O_3 for 1 hr. $^{-/-}$ and $^{+/+}$ refer to the *pml* background of the transformed cells. The scale bar represents 5 μm. The bottom row shows localization of SUMO-1, SUMO-2, or daxx, after 1 hr exposure to As_2O_3 of PML/RARA-transformed $pml^{-/-}$ progenitors.

(C) As_2O_3-induced PML/RARA degradation in $pml^{-/-}$ or $pml^{+/+}$ transformed MEF cells.

(D) Schematic model of As_2O_3-induced PML/RARA trafficking.

PML/RARA Oxidation Triggers Its Matrix-Targeting, NB Reformation, and APL Remission

Ex vivo, As_2O_3 and its FLAsH analog induced PML/RARA hypersumoylation (Figure 2A). These arsenicals, as well as H_2O_2, elicited intermolecular disulfide-bound PML/RARA multimer formation, in both transduced MEF cells and murine APL blast cells (Figure 2A). PML/RARA multimers became matrix-associated upon As_2O_3 treatment. As_2O_3 also induced the coalescence of PML/RARA microspeckles (together with SUMO-1/2/3 and daxx) into typical NBs in either $pml^{+/+}$ or $pml^{-/-}$ transduced hemopoietic progenitors or fibroblasts (Figure 2B). Whereas the kinetics of NB reformation were delayed in $pml^{-/-}$ cells, PML/RARA was ultimately fully degraded upon As_2O_3 treatment in both settings (Figure 2C). These data demonstrate that As_2O_3-induced PML/RARA multimerization results in its targeting to the matrix, drives NB reformation and ultimately allows its sumoylation/degradation, in the same manner as for PML (Figure 2D).

(F) CHO cells were transiently transfected with $(His)_{x6}$-PML-V and/or CFP-PML-III. Total extracts in nonreducing (top) or reducing (bottom) conditions were labeled with the indicated antibodies. The arrows indicate the different PML complexes, multimers are denoted $(PML)_2$. The arrowheads indicate mixed PML-III/PML-V multimer.

(G) As_2O_3 treatment increases PML multimerization in transiently transfected CHO cells.

(H) Stably expressed PML exhibits oxidant stress-mediated multimerization. PAO and FlAsH are 10^{-6} M, As203 and CdCl2 are 10^{-5} M, and H202 is 10^{-3} M. Treatment for 1 hr. PML monomers, multimers, and SUMO/ubiquitin conjugates (unambiguously identified in previous studies (Lallemand-Breitenbach et al., 2008), are indicated.

(I) Pretreatment of PML-transfected CHO cells with NEM for 1 hr inhibits As_2O_3- or H_2O_2-induced PML multimer formation. The asterisk denotes a nonspecific protein. Analysis was performed in nonreducing conditions.

(J) Pretreatment of PML-transfected CHO cells with NAC for 3 days inhibits As_2O_3- induced PML dimerization.

(K) NB formation in vivo after paraquat or As_2O_3 treatment of nude mice xenografted with CHO-PML cells. Top left panels show tumor sections stained with anti-PML (red) and anti-daxx (green) antibodies. The scale bar represents 10 μm. A zoom (2×) on representative cells is shown in the top right panels. Bottom panels show bone marrow cells with endogenous pml. The scale bar represents 5 μm.

(L) Western blot analysis of CHO-PML xenografts after in vivo As_2O_3 or paraquat exposure. Arrowheads indicate covalent PML multimers. See also Figure S1.

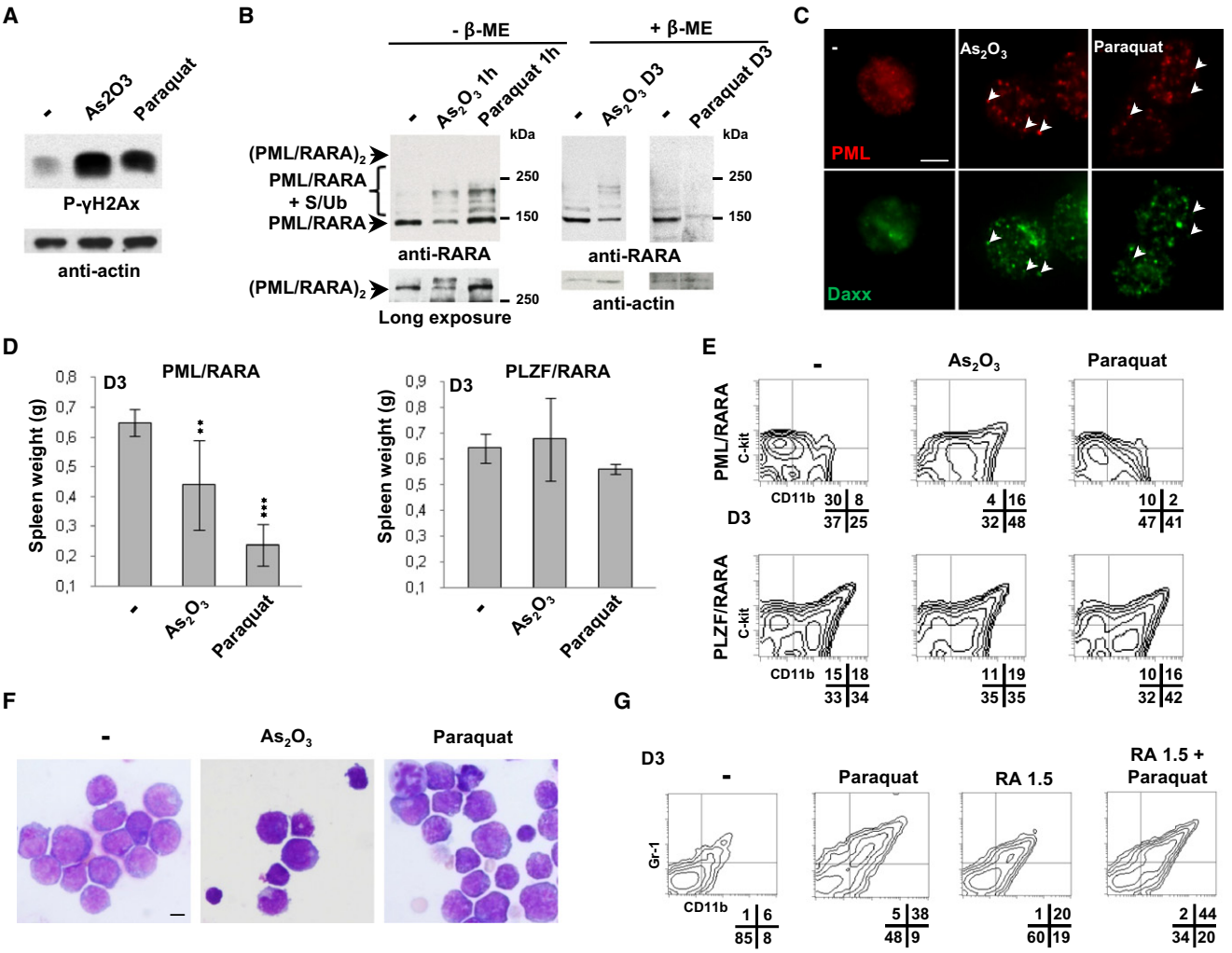


Figure 3. PML/RARA Oxidation Promotes APL Response In Vivo

(A) Treatment of mice with paraquat or As_2O_3 for 2 days induce oxidative stress and γ-H_2AX phosphorylation in APL bone marrow cells.

(B) Treatment of APL mice with oxidants (As_2O_3 or paraquat) induces PML/RARA multimerization, sumoylation (1 hr, left panel) and degradation (3 days, right panel) in APL bone marrow cells. Presence of β-ME is indicated. A long exposure of the gel, allowing detection of the $(PML)_2$ species after a 1 hr exposure to oxidants, is shown below.

(C) NB reformation in bone marrow cells of APL mice treated for 3 hr with As_2O_3 or Paraquat. Immuno-fluorescence with anti-daxx or hPML antibodies to detect hPML/RARA. The scale bar represents 5 μm.

(D) Tumor regression induced by paraquat or As_2O_3 is specific for PML/RARA-driven APL. Spleen weight of the two types of APL mice at 3 days. Mean ± SD of three independent experiments is shown. $^{**}p < 0.01$; $^{***}p < 0.001$.

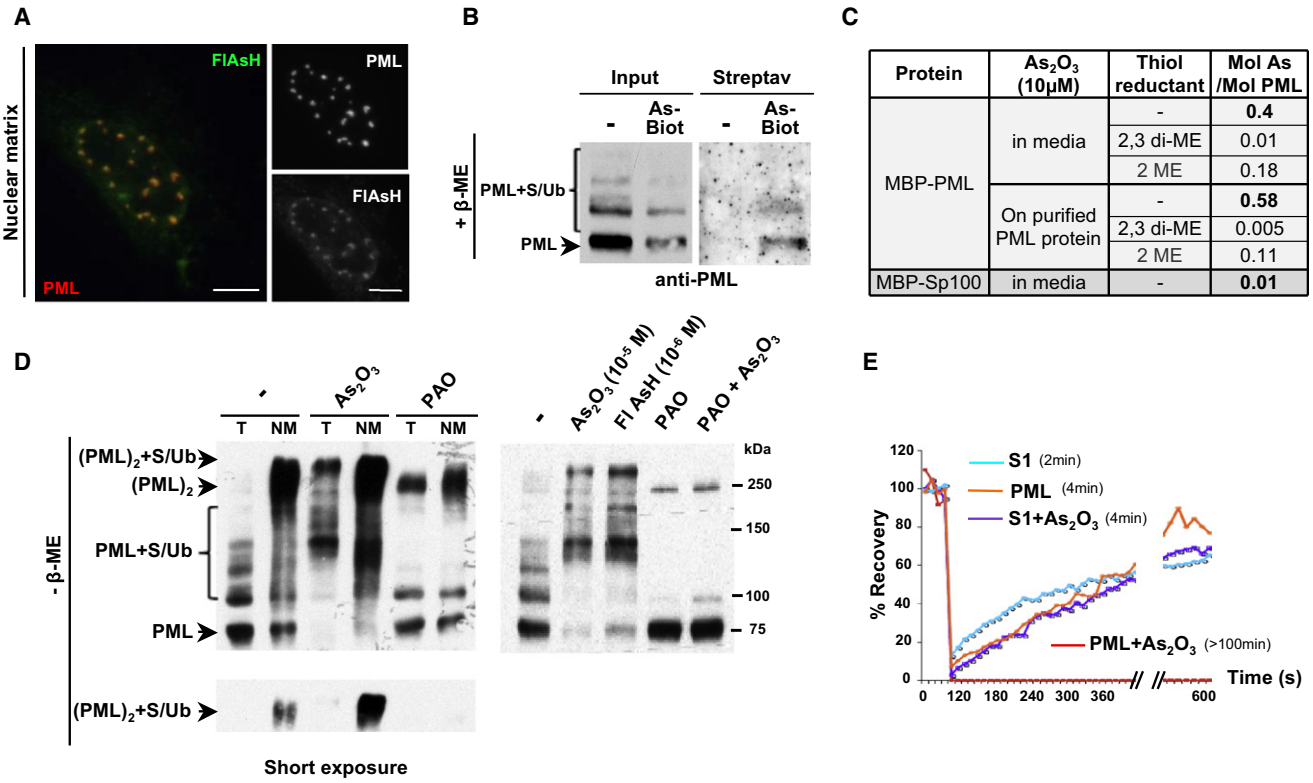
(E) FACS analysis of bone marrow cells of APL mice treated or not treated for 3 days with As_2O_3 or paraquat. Both PML/RARA- and PLZF/RARA-driven murine APLs are shown. The antibodies used and the cell distributions are indicated.

(F) May Grünwald Giemsa staining of bone marrow cells of PML/RARA APL mice treated with As_2O_3 or paraquat for 3 days.

(G) Same as in (E) with treatment with suboptimal doses of RA that fails to significantly differentiate (Nasr et al., 2008).

We then treated APL mice with paraquat or As_2O_3. Both agents induced oxidative stress in bone marrow APL cells, as measured by phospho-γH_2AX (Figure 3A). Oxidation by paraquat was generally more pronounced than that induced by As_2O_3, as measured by homocysteine formation and DCF-HDA labeling (not shown). After 1 to 3 hr in vivo treatment, both agents induced PML/RARA multimerization and sumoylation, which were accompanied by NB reformation (Figures 3B and 3C). At day 3, both treatments led to PML/RARA loss in the APL blasts (Figure 3B).

Critically, both paraquat and As_2O_3 triggered a dramatic APL regression, as assessed by spleen weight (Figure 3D), although the bone marrow remained essentially leukemic at day 3. Consistent with the greater oxidative stress, paraquat was generally more active than As_2O_3 in eliciting NB reformation and APL regression. In animals treated with either agent, PML/RARA degradation was accompanied by loss of Kit expression (a marker commonly expressed by early clonogenic progenitors) and an increase in CD11b expression (Figure 3E). Yet, at this time point, As_2O_3 or paraquat treatments were not accompanied by



Protein	As_2O_3 (10μM)	Thiol reductant	Mol As /Mol PML
MBP-PML	in media	-	**0.4**
		2,3 di-ME	0.01
		2 ME	0.18
	On purified PML protein	-	**0.58**
		2,3 di-ME	0.005
		2 ME	0.11
MBP-Sp100	in media	-	**0.01**

Figure 4. As_2O_3 Binds Directly to PML

(A) Fluorescent diarsenical labels PML bodies in in situ-prepared nuclear matrix of CHO-PML cells. The scale bar represents 5 μm.

(B) Streptavidin pull-down shows that As-biotin binds to PML in CHO-PML-treated cells.

(C) Direct binding of As_2O_3 on bacterially produced full-length MBP-PML. MBP-Sp100 is a negative control. Arsenic content was measured by atomic absorption.

(D) Shown on the left are stable CHO-PML cells after treatment with As_2O_3 or PAO for 1 hr. Shown on the right is a comparison of As_2O_3 (10^{-5}M), FlAsH (10^{-6}M), and PAO (10^{-5}M) in the same cells.

(E) FRAP analysis of CFP-PML and YFP-SUMO-1 exchange rates on NBs of transduced MEF *pml*$^{-/-}$ cells treated or not with 10^{-6} M As_2O_3. See also Figure S2.

morphologically detectable differentiation (Figure 3F), as previously shown for As_2O_3 (Lallemand-Breitenbach et al., 1999).

As_2O_3 does not affect APL driven by the variant PLZF/RARA fusion (Rego et al., 2000). Critically, paraquat did not impede growth, nor did it induce Kit loss or CD11b induction of these variant APLs (Figures 3D and 3E). Thus, in vivo, paraquat specifically targets PML/RARA, rather than inducing nonspecific toxicity. In classical PML/RARA-driven APLs, suboptimal doses of retinoic acid (RA, 1.5 mg) induce transient differentiation, but do not affect LIC self-renewal, because of incomplete PML/RARA degradation (Nasr et al., 2008). Consequently, upon 3 days of this RA treatment, marrow cells retained an immature phenotype. When combined with RA 1.5, paraquat enhanced granulocytic differentiation (Figure 3G) and APL regression (not shown), as previously shown for As_2O_3 (Nasr et al., 2008). Taken together, our finding that paraquat mimics the biological effects of As_2O_3 on PML and PML/RARA (oxidation, NB formation, partner recruitment, degradation, and APL regression) implies that ROS production contributes to As_2O_3 therapeutic activity in PML/RARA-driven APL.

Arsenic Binds to PML

PML is a cysteine-rich protein with a dicysteine motif (C212/C213), which could directly bind As_2O_3. Synthetic FlAsH diarsenical cooperatively binds two CC motifs (Luedtke et al., 2007), upon which it becomes fluorescent (Griffin et al., 2000). Using FlAsH, labeling of PML NBs was distinctly observed in CHO-PML cells and even more prominently in in situ nuclear matrix preparations (Figure 4A). To determine whether PML directly binds to arsenic, we exposed CHO-PML cells to an arsenic-biotin (As-biotin) derivative. As-biotin conjugated proteins were purified on a streptavidin column and eluates analyzed by western blotting with anti-PML antibodies. Only cells exposed to As-biotin yielded detectable PML proteins (Figure 4B). Finally, we quantified by atomic absorption arsenic binding on purified MBP-PML produced in bacteria. We detected arsenic atoms (on average 0.5 arsenic atoms per polypeptide) when 10 μM As_2O_3 was added directly onto the purified MBP-PML proteins or even in culture media (Figure 4C). Arsenic binding was fully reversed by dithiols, but much less by monothiols. Another NB-associated protein, Sp100, did not bind arsenic atoms.

FlAsH diarsenical initiated PML sumoylation and degradation more efficiently than As_2O_3, which may bind a single or two CC pairs (Figure 4D). That FlAsH became fluorescent (Figure 4A) suggests that it actually binds two CC pairs. Conversely, the bulky monoarsenical oxidant phenylarsine oxide (PAO), which only binds a single CC pair, promoted some PML disulfide formation (presumably through generation of ROS), yet it

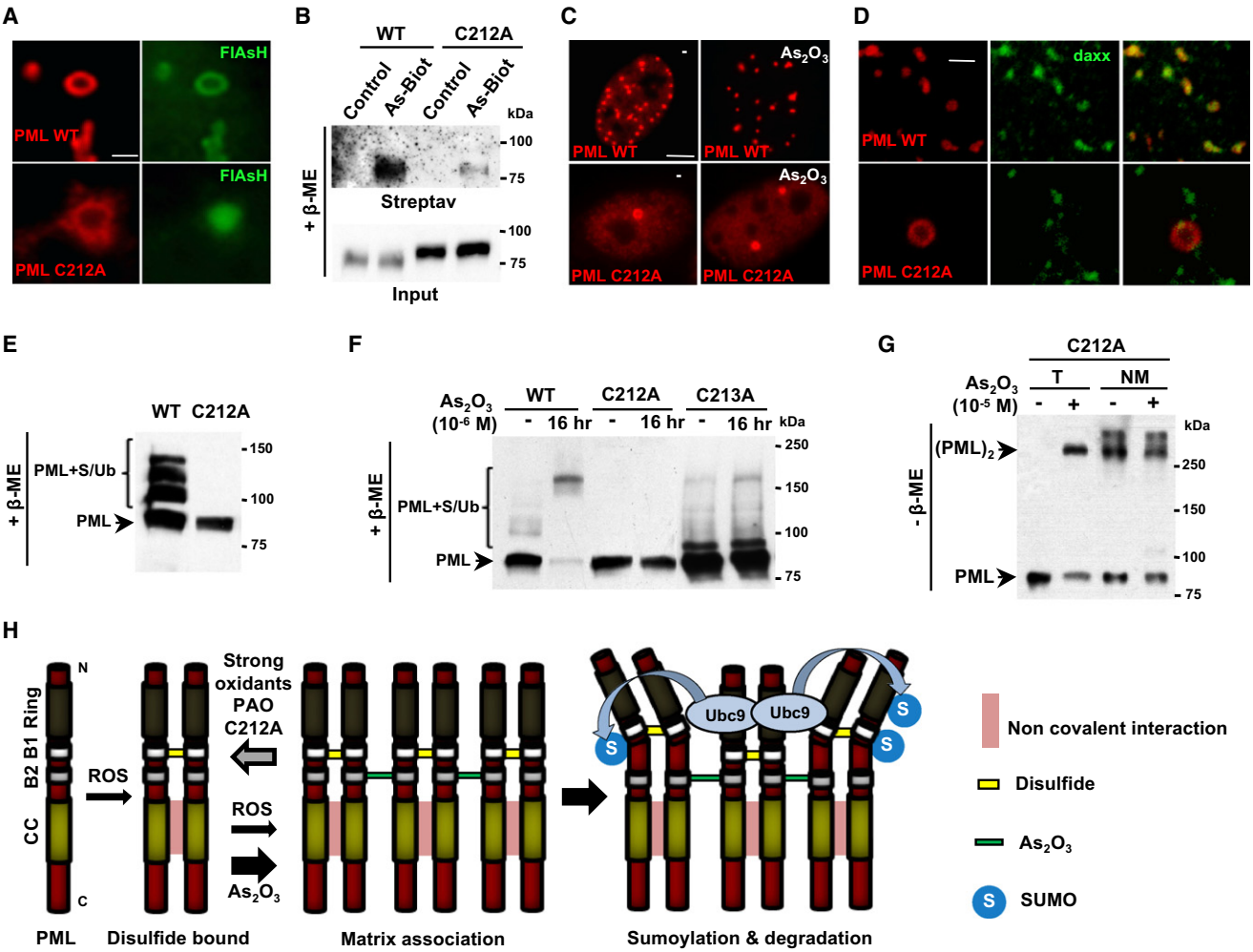


Figure 5. C212 Is Required for PML As_2O_3 Binding and Basal Sumoylation

(A) Bodies generated by PMLC212A expression no longer bind FlAsH (green). Zoom on a single NB is shown. The scale bar represents 1 μm.

(B) PMLC212A no longer binds As-biotin in transfected COS cells.

(C) PMLC212A displays altered basal nuclear distribution and its diffuse nuclear fraction is insensitive to As_2O_3. The scale bar represents 5 μm.

(D) Stably expressed PMLC212A does not recruit daxx. Zoom within the nucleus. The scale bar represents 1 μm.

(E) Absence of basal sumoylation of stably expressed PMLC212A.

(F) PMLC212A or PMLC213A are resistant to As_2O_3-induced hypersumoylation and degradation after overnight As_2O_3 treatment of stable CHO transfectants.

(G) As_2O_3 induces PMLC212A disulfide-bound multimer formation, but does not enhance matrix-association in stable transfectants. T, total extract; NM, matrix-preparation. The nuclear matrix extracts correspond to ten times as many cells as the total extracts.

(H) A sequential model for the formation of PML NBs, showing the progressive association of PML to the matrix and the resulting sumoylation. See also Figure S3.

paradoxically antagonized both basal PML matrix attachment and sumoylation (Figure 4D; Figure S2). Moreover, PAO completely abolished As_2O_3 effects on PML sumoylation at equimolar concentrations. These unexpected findings could support the idea that two arsenic atoms, but not one, may crosslink two PML polypeptides, mimicking disulfides (Luedtke et al., 2007) and promoting sumoylation.

We then assessed PML exchange rates on NBs by FRAP. In the absence of As_2O_3, PML was dynamically associated with NBs, in keeping with previous studies (Figure 4E) (Boisvert et al., 2001; Weidtkamp-Peters et al., 2008). Exposure to As_2O_3 elicited complete immobilization of CFP-PML, but not of YFP-SUMO-1 (which may be conjugated to many other NB-associated proteins). Such dramatic immobilization most likely directly reflects PML crosslinking by disulfide formation and/or arsenic binding.

C212 Contributes to Response to As_2O_3

The C212/C213 motif may be directly implicated in arsenic binding. The outer PML-labeled shell formed by the PMLC212A, C213A, or C212/213A mutants lost the FlAsH labeling observed in normal NBs. Unexpectedly, the PML-negative inner core accumulated FlAsH in the mutant (Figure 5A), an observation that deserves further exploration. PMLC212A similarly exhibited sharply diminished As-biotin binding (Figure 5B). Thus the PML C212/213 dicysteine motif contributes to direct arsenic binding.

Contrasting with wild-type PML, stable expression of PMLC212A or PMLC213A mutants in CHO cells resulted in

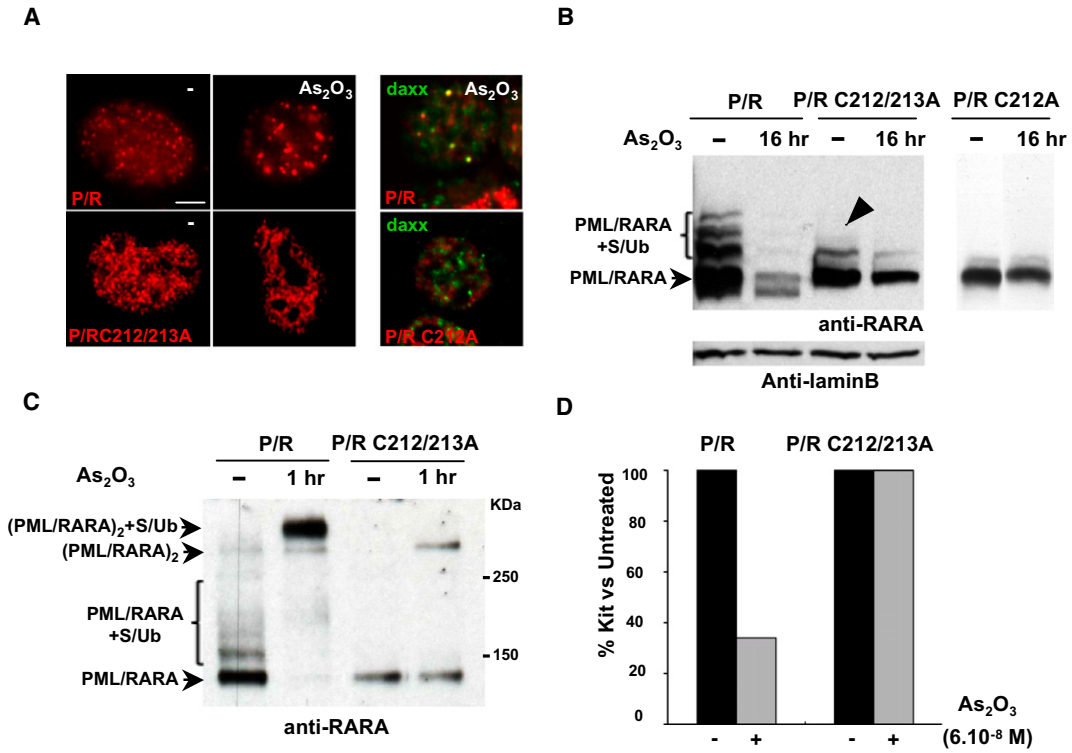


Figure 6. C212 Is Required for the Effect of As_2O_3 on PML/RARA

(A) PML/RARAC212A- or PML/RARAC212/213A-transformed progenitors are resistant to As_2O_3-induced NB reformation (left) and fail to recruit endogenous daxx (green) (right). The scale bar represents 5 μm.

(B) Shown on the left is degradation of PML/RARA and As_2O_3-binding mutants after As_2O_3 treatment in hematopoietic progenitors. Arrowhead: change in the basal sumoylation pattern. Shown on the right is As_2O_3 insensitivity of PML/RARAC212A in MEFs.

(C) Comparison between PML/RARA and PML/RARAC212/213A covalent multimerization upon As_2O_3 treatment in transduced MEFs.

(D) Loss of Kit expression in methyl-cellulose clonogenic cultures. Data are from a representative experiment out of three similar ones.

strong diffuse nuclear PML labeling with only one or two abnormally large NBs. Importantly, those failed to accumulate daxx and SUMO (Figures 5C and 5D), although they still recruited GFP-UBC9, probably through PML RING (Figure S3). The PMLC212A and PMLC213A mutants both exhibited an altered basal or As_2O_3-induced sumoylation (Figure 5E) and failed to be degraded (Figure 5F). These two PML mutants thus mimic inactivation of the key K160 sumoylation site. Whereas the diffuse nuclear fraction of the wild-type PML protein was entirely targeted onto NBs upon As_2O_3 exposure (Lallemand-Breitenbach et al., 2001; Zhu et al., 1997), that of PMLC212A was essentially As_2O_3 insensitive (Figure 5C). This mutant was also defective for As_2O_3-enhanced association with the nuclear matrix (Figure 5G). It nevertheless showed some basal matrix-association and As_2O_3-enhanced disulfide-mediated crosslinking. Altogether, these data indicate that C212 contributes not only to direct As_2O_3 binding, but also to basal NB biogenesis and PML sumoylation. Mutations of neighboring C148, C151, or C227 cysteines did not affect matrix association, NB biogenesis, FlAsH binding, or As_2O_3-induced degradation (not shown). Collectively, our findings converge on a model for NB assembly, integrating both the initial role of ROS-mediated PML dimerization and direct arsenic-binding, to ultimately control PML sumoylation (Figure 5H, see Discussion).

Mutant PML/RARAC212/213A or C212A retained the ability to transform primary hemopoietic progenitors in a methyl-cellulose replating assay, at least up to the third passage, like the wild-type PML/RARA oncoprotein. These mutants formed nuclear microspeckles (Figure 6A), which, as expected, were not labeled by FlAsH (not shown). For both mutants, basal PML/RARA sumoylation was altered (Figure 6B). Similarly, NB reformation, enhanced sumoylation, daxx recruitment, and PML/RARA degradation upon As_2O_3 exposure were all lost for both mutants (Figures 6A and 6B), again mimicking PML/RARAK160R (Zhu et al., 2005). Moreover, multimer formation was significantly decreased upon As_2O_3 exposure (Figure 6C). Finally, PML/RARAC212/213A- or PML/RARAC212A-transformed progenitors were completely resistant to As_2O_3-induced loss of Kit expression (Figure 6D), a surrogate for the response to arsenic in vivo (Lallemand-Breitenbach et al., 2008). Thus, C212 is required to elicit As_2O_3-induced PML/RARA degradation and, ultimately, APL response to As_2O_3.

DISCUSSION

By implicating ROS-mediated PML oxidation and direct As_2O_3 binding in therapy response, our results provide mechanistic insights into the basis of APL cure by As_2O_3 and have broad implications for the physiological control of NB formation.

We first demonstrate that ROS regulate NB-biogenesis in vivo, explaining the abundance of NBs in multiple stress conditions or in cells exposed to high oxygen concentrations, such as endothelial cells (Koken et al., 1995; Lallemand-Breitenbach and de Thé, 2010). NBs are nuclear matrix domains and disulfides were previously implicated in nuclear matrix formation (Kaufmann et al., 1991; Stuurman et al., 1992b). PML is the first example of a protein organizing a nuclear domain in a ROS-dependent manner, suggesting that PML may be a ROS sensor. Both ROS and PML have been implicated in multiple biological processes, notably DNA damage response, senescence, and stem cell self-renewal, as well as in the fine-tuning of some critical signaling pathways, including HIF1a or PTEN/AKT (Song et al., 2008; Trotman et al., 2006; Bernardi and Pandolfi, 2007; Ito et al., 2008; Pearson et al., 2000). PML NB formation could thus mediate some effects of basal ROS. In cellulose synthase, cysteines arranged in a zinc finger become engaged into multiple intermolecular disulfides upon ROS exposure (Kurek et al., 2002). This ROS-induced, oxidation-mediated, transition is responsible for the action of herbicides on cellulose synthase activity. PML, which harbors three zinc fingers, forms several disulfide bridges (data not shown), likely all required for full matrix association and PML sumoylation. This could explain why, despite formation of some disulfide bridges, the C212A mutant exhibits a defective sumoylation. Matrix association and/or sumoylation might also require an interchain zinc finger (Callaghan et al., 2005), itself possibly involving C212. Future studies should identify all cysteines involved in ROS-sensitive PML dimerization, define their connectivity, and elucidate the structural details of multimer formation.

Arsenic elicits both PML association with the nuclear matrix and its sumoylation (Lallemand-Breitenbach et al., 2001). These are presumably two consecutive steps, because mutants that do not associate with the nuclear matrix are not sumoylated, whereas nuclear matrix-associated PML is consistently entirely polysumoylated (Figure 4D) (Lallemand-Breitenbach et al., 2001). This suggests the following model to explain NB-biogenesis and PML sumoylation (Figure 5H): basal, As_2O_3- or paraquat-induced ROS promote disulfide-mediated covalent bridging of preassembled noncovalent PML or PML/RARA dimers, allowing them to multimerize, acquire nuclear matrix features, and thus form primary NBs. These multimers are subsequently sumoylated. SUMO conjugation of PML in *trans* by the PML RING-bound UBC9, rather than in *cis* (similarly to receptor tyrosine kinase *trans*-phosphorylation), could explain why matrix-associated PML mesh is so efficiently sumoylated. This sequential model accounts for the observation that NAC or NEM blunts NB formation (Figure 1) and As_2O_3 response in APL cells (Miller et al., 2002). Yet, other mechanisms may coexist and cooperate with the latter to enforce As_2O_3-triggered PML sumoylation. Indeed, As_2O_3 or FlAsH directly bind to PML and may trigger sumoylation of PML or PML/RARA monomers (Figures 1H and 4D, for example), either by enhancing UBC9 binding onto PML (Zhang et al., 2010) or by forming As_2O_3-mediated intramolecular bridges (Luedtke et al., 2007). Biarsenicals could also contribute to formation of intermolecular PML crosslinks. In that respect, the complete immobilization of PML NBs upon As_2O_3 in FRAP experiments, strongly argues for a distinct, tighter, type of PML association. Structural and physicochemical studies should define the arrangement of arsenic atoms within PML multimers and determine the functional consequences of binding. The sumoylation pathway, which relies on some thiol enzymes, is highly ROS sensitive (Bossis and Melchior, 2006; Han et al., 2010). PML sumoylation allows NBs to sequester many partner proteins (Lallemand-Breitenbach and de Thé, 2010), suggesting that ROS could trigger partner sequestration. Given the proposed role of PML as a SUMO E3 ligase (Quimby et al., 2006) and the fact that most partners may themselves undergo sumoylation, PML NB aggregation could contribute to their stress-regulated sumoylation (Saitoh and Hinchey, 2000).

Our data suggest that As_2O_3-induced ROS and direct binding both contribute to the curative effect of As_2O_3 in APL. ROS primarily control NB formation (and indirectly sumoylation) in vivo, whereas As_2O_3 dramatically enhances sumoylation both ex vivo and in vivo. Critically, nonarsenical ROS-inducers (paraquat, a-TOS; Freitas et al., 2009) induce PML/RARA degradation, dramatic regressions, and even promote long-term survival in murine APLs (Figure 3 and E. Rego, personal communication). Iron deprivation-induced ROS similarly initiates apoptosis and differentiation in APL cells (Callens et al., 2010). This strongly suggests that As_2O_3-induced ROS significantly contribute to therapy response in vivo. Conversely, there is circumstantial evidence that direct As_2O_3 binding is also important, as in vivo, we detected oxidant-induced sumoylation of PML/RARA with oxidative stresses significantly greater than those imposed by As_2O_3. Such higher clinical efficiency of As_2O_3 could reflect its ability to bind to an exquisitely sensitive site in the protein, which controls its sumoylation, and to crosslink PML, allowing tight matrix association.

That As_2O_3-induced ROS significantly contribute to its clinical efficacy, suggests that human APL may be susceptible to a variety of other ROS-inducers. In that respect, the standard APL chemotherapy, anthracyclines, induces massive ROS production (Berthiaume and Wallace, 2007). When used in combination with RA, anthracyclines dramatically enhances its long-term efficacy, in the same manner as As_2O_3 (Lallemand-Breitenbach et al., 1999; Nasr et al., 2008; Wang and Chen, 2008). Anthracyclines-induced ROS may thus contribute to their antileukemia effects. Conversely, lack of ROS sensitivity of PLZF/RARA-driven APLs, together with partial RA-resistance (Nasr et al., 2008), may account for their resistance to RA/anthracycline therapy (Licht et al., 1995). Collectively, our studies identify ROS as critical regulators not only of PML NB biogenesis, but also of PML/RARA degradation, making an essential contribution to APL cure by As_2O_3. Future studies should establish the respective contribution of ROS induction and direct binding in APL response to As_2O_3, by identifying mutations uncoupling these two phenotypes and testing their in vivo importance for As_2O_3 response.

EXPERIMENTAL PROCEDURES

Antibodies

Homemade rabbit and chicken anti-hPML antibodies were used as described (Lallemand-Breitenbach et al., 2008). PML isoform-specific antibodies have been previously characterized (Condemine et al., 2006). Anti-mouse pml monoclonal was from Upstate Biochemicals. Rabbit polyclonal anti-RARA115 was a kind gift of Pierre Chambon. The rabbit polyclonal anti-RXRA (D20) and anti-daxx (M-112), and goat polyclonal anti-LaminB (M-20) antibodies were from Santa Cruz Biotechnology. Anti–SUMO-1 (GMP-1) and

rabbit polyclonal anti-SUMO-2/3 antibodies were from Zymed Laboratories. Anti-phospho-γH_2AX (rabbit 20E3) was from Cell Signaling. All primary antibodies were revealed by AlexaFluor 488 or 594-labeled secondary antibodies from Molecular Probes.

Cell Lines and Treatments

CHO, MRC5, COS, and *pml*$^{-/-}$ or *pml*$^{+/+}$ MEF cells were grown in 10% fetal calf serum (FCS)-supplemented DMEM medium (GIBCO). Primary mouse APL blast cells were obtained from the bone marrow of serially transplanted mice (Nasr et al., 2008) and cultured in RPMI medium supplemented with 10% FCS, IL-3, IL-6, and SCF (GIBCO). CHO-PML cells stably express PMLIII or the indicated mutants.

As_2O_3 (Fluka) treatment was performed as indicated at 1 μM or 10 μM for 1 hr, or at 1 μM for 16 hr to induce PML degradation. PML/RARA degradation was induced by a 16 hr 0.1 μM As_2O_3 treatment of *pml*$^{-/-}$ MEF cells and an 8-day 6×10^{-8} M treatment of methylcellulose cultured transformed mouse progenitors. CHO cells were treated with 1 μM TC-FlAsH (FlAsH, Molecular Probes), 10 μM $CdCl_2$ (Fluka), or 10 μM PAO (Sigma) for 1 hr, or with 1 mM H_2O_2 (Sigma) for 30 min. Dithiarsolan-biotin conjugate (As-biotin) was kindly given by Kenneth L. Kirk and used at 10 μM for 1 hr. Cells were pretreated with 100 μM NEM (Sigma) for 1 hr before adding 10 μM As_2O_3 or 1 mM H_2O_2. Buffered NAC was used at a concentration of 1 mM.

Western Blot Analysis, In Situ Nuclear Matrix Preparations

For all PML extractions, NEM (10 mM) was added to the lysis buffer to prevent artifactual de novo formation of disulfides. For analysis under non-reducing conditions, β-Mercaptoethanol was omitted from the standard Laemmli buffer. Cell lysates were resolved on 7% or 3 to 10% gradient SDS-PAGE gels. Nitrocellulose membranes were blocked in 5% milk, incubated with specific antibodies and detected with the SuperSignal WestPico (Pierce) chemiluminescent substrate.

In situ nuclear matrices were prepared as described (Stuurman et al., 1990) and used for immunofluorescence or resuspended in Laemmli buffer for western blot analysis.

Animal Experiments

Mouse experiments were repeated three times. Animals were handled according to the guidelines of institutional animal care committees, using protocols approved by the "Comité Régional d'Ethique Expérimentation Animale (CREEA) n°4." Paraquat (Sigma, 25 mg/kg) was injected intraperitoneally daily in PML/RARA APL mice treated or not with RA 1.5 mg slow-release pellets (Innovative Research of America) for 3 or 5 days. Arsenic was delivered as previously described (Nasr et al., 2008). APL cells were obtained from the bone marrow and spleen (Nasr et al., 2008). Flow cytometry determination of differentiation-associated surface antigens (Mac1/Gr1/Kit) was performed as reported (Nasr et al., 2008). PLZF/RARA+RARA/PLZF-driven APL were obtained from P.P. Pandolfi and propagated exactly as previously described (Nasr et al., 2008).

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, three figures and one table and can be found with this article online at doi:10.1016/j.ccr.2010.06.003.

ACKNOWLEDGMENTS

The laboratory is supported by the Ligue Nationale contre le Cancer, INSERM, CNRS, University Paris Diderot (formerly known as Paris-7), the Institut Universitaire de France, EPITRON (an integrated project funded by the European union under the 6th framework program (LSHC-CT-2005-518417)), and Canceropole programs. B.R. was supported by the Leukemia and Lymphoma Society of Canada. M. Jeanne was supported by a doctoral fellowship of the Ile de France Region. We thank J. Godet and the Comité des Yvelines de la Ligue contre le Cancer for their continuous trust and support. We are grateful to P.G. Pelicci for informal discussions about PML disulfides and to Z. Chen for reciprocal exchanges of unpublished observations. We thank K. Kenneth and J. Heredia (supported by the NIDDK, NIH intramural research program) for providing As-biotinylated, P.P. Pandolfi for pml-/- mice, P. Hemmerich for advice on FRAP analysis, and C. Bas for atomic absorption analysis. We thank M. Pla and the animal house facility staff for their help, C. Dargemont and J.C. Chottard for helpful suggestions. We warmly thank N. Setterblad for his dedication to the project and J. Ablain, G. Ghysdael, and F. Sigaux for critical reading of the manuscript. This work was presented at Karolinska Institutet in August 2009 and in the 5th APL meeting in Rome in September 2009.

Received: March 10, 2010
Revised: May 5, 2010
Accepted: June 10, 2010
Published: July 12, 2010

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